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The Human Immunodeficiency Virus Type 1 Vpu Protein Inhibits NF-κB Activation by Interfering with βTrCP-mediated Degradation of IκB*

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The human immunodeficiency virus type 1 (HIV-1) Vpu protein binds to the CD4 receptor and induces its degradation by cytosolic proteasomes. This process involves the recruitment of human βTrCP (TrCP), a key member of the SkpI-Cdc53-F-box E3 ubiquitin ligase complex that specifically interacts with phosphorylated Vpu molecules. Interestingly, Vpu itself, unlike other TrCP-interacting proteins, is not targeted for degradation by proteasomes. We now report that, by virtue of its affinity for TrCP and resistance to degradation, Vpu, but not a phosphorylation mutant unable to interact with TrCP, has a dominant negative effect on TrCP function. As a consequence, expression of Vpu in HIV-infected T cells or in HeLa cells inhibited TNF-α-induced degradation of IκB-α. Vpu did not inhibit TNF-α-mediated activation of the IκB kinase but instead interfered with the subsequent TrCP-dependent degradation of phosphorylated IκB-α. This resulted in a pronounced reduction of NF-κB activity. We also observed that in cells producing Vpu-defective virus, NF-κB activity was significantly increased even in the absence of cytokine stimulation. However, in the presence of Vpu, this HIV-mediated NF-κB activation was markedly reduced. These results suggest that Vpu modulates both virus- and cytokine-induced activation of NF-κB in HIV-1-infected cells.

The human immunodeficiency virus type 1 (HIV-1)[1] is a complex retrovirus, which, in addition to the prototypical Gag, Pol, and Env products, requires the activity of a number of accessory proteins for replication *in vivo*. Most if not all of the viral accessory proteins exert multiple independent functions. This includes Vpu, which not only regulates virus release from infected cells (1, 2) but also induces degradation of the HIV-1 receptor CD4 in the endoplasmic reticulum (3).

The best characterized function of Vpu is its ability to induce the degradation of CD4 (for reviews, see Refs. 4 and 5). Structurally, this activity is dependent on sequences present in the cytoplasmic domain of Vpu and, in particular, requires the phosphorylation by casein kinase II of two highly conserved serine residues at positions 52 and 56 (6). CD4 degradation is initiated by physical interactions between the cytoplasmic domains of Vpu and CD4 (7). Interestingly, phosphorylation of the two serine residues in Vpu, while critical for CD4 degradation, is not required for this initial binding (7). Instead, the phosphoserine residues are involved in the recruitment of human βTrCP (TrCP). TrCP is a key component of a recently characterized E3 ubiquitin ligase complex that regulates protein degradation through the ubiquitin-dependent proteasome pathway (8). This finding was of particular significance, since there is evidence for the involvement of the ubiquitin-proteasome machinery in Vpu-mediated CD4 degradation (9, 10). The role of TrCP in the SkpI-Cdc53-F-box protein (SCF) ubiquitin ligase complex is to select and recruit the proper substrate for polyubiquitination by the SCF. This is achieved by the modular organization of TrCP, which allows for simultaneous interactions with the target protein on the one hand through its C-terminal WD repeats and the SCF on the other hand through interactions between its F-box domain and SkpI (reviewed in Ref. 11).

Recent studies have confirmed the important role of TrCP in the regulated degradation of cellular proteins. Indeed, the SCF^{TrCP} was shown to mediate the ubiquitination and proteasome targeting of β-catenin as well as the NF-κB-inhibitory molecule IκB-α (12–14). Similar to the TrCP-binding domain in Vpu (15), the signal for recognition of both β-catenin and IκB by TrCP includes a pair of conserved phosphorylated serine residues that are arranged in a consensus motif, $DS[PO_3]G\psi XS[PO_3]$ (where ψ represents a hydropholic residue) present in all three proteins. Serine phosphorylation plays a major role in regulating the stability of SCF target proteins. In the case of IκB-α, activation of the IκB kinase complex following stimulation by cytokines such as TNF-α results in the phosphorylation of two serine residues (Ser^{32} and Ser^{36}) and leads to the rapid degradation of IκB-α by cytosolic proteasomes (16, 17). Similarly, β-catenin phosphorylation constitutes a signal for degradation and is developmentally regulated by glycogen synthase kinase 3β (18).

Among the known substrates of TrCP, Vpu is exceptional in that it does not appear to be targeted for degradation itself but rather acts as an adapter for proteasome targeting of CD4 (8). Also, in contrast to NF-κB or β-catenin, Vpu is constitutively phosphorylated by the ubiquitous casein kinase II (19), and, as a consequence, the vast majority of Vpu present in infected cells is TrCP binding-competent at all times. Both the constitutive Vpu phosphorylation as well as the inability to degrade TrCP-bound Vpu are likely to contribute to unusually stable complexes between the two proteins. Indeed, we have previously reported that expression of Vpu and TrCP *in vitro* in the

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[1] The abbreviations used are: HIV-1, human immunodeficiency virus type 1; TrCP, human βTrCP; SCF, SkpI, Cdc53, F-box protein; TNF, tumor necrosis factor; PCR, polymerase chain reaction; bp, base pair; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; IL, interleukin.

presence of microsomal membranes resulted in efficient redistribution of TrCP from its normal cytoplasmic fraction to the Vpu-containing membrane fraction (8).

In HIV-infected cells, Vpu is synthesized from a bicistronic mRNA also encoding the viral envelope (Env) protein gp160. Comparative analysis of Vpu and Env synthesis suggests that the two proteins are synthesized at similar rates. However, unlike the Env protein, which is packaged into virions and exported from the cell, Vpu remains largely cell-associated and accumulates over time in infected cells. Based on these observations, we speculated that the accumulation of Vpu in HIV-infected cells could interfere with the normal physiological function(s) of TrCP by acting as a competitive inhibitor of TrCP. To test this hypothesis, we characterized in this study the effects of Vpu on TrCP-mediated degradation of IkB-α and the resulting activation of NF-κB transcriptional activity. NF-κB is a transcriptional activator that under resting conditions resides in the cytoplasm in an inactive complex with its inhibitor IκB-α. Degradation of IkB-α following stimulation of cells by growth factors, chemokines, or inflammatory cytokines such as TNF-α leads to activation of NF-κB and its translocation into the nucleus, where it induces target gene expression. NF-κB plays a central role in the expression of numerous genes encoding cytokines, chemokines, and factors involved in T-cell activation and proliferation (reviewed in Refs. 17 and 20). In addition, NF-κB has been implicated in the control of antiapoptotic genes (reviewed in Refs. 21 and 22). Thus, deregulation of NF-κB in HIV-infected cells will impact on a multitude of normal cellular functions.

We now present evidence that the stable interaction between Vpu and TrCP in HIV-1-infected cells leads to impaired TrCP function. Indeed, infection of the CD4-positive A3.01 cell line with the NL4-3 molecular clone of HIV-1 but not with its Vpu-deficient variant resulted in an inhibition of TNF-α-induced IκB degradation. Using CD4/Vpu chimeric molecules with biological activities indistinguishable from those of authentic Vpu, we demonstrate that Vpu is required and sufficient to block IκB degradation. Using a Vpu phosphorylation mutant, we confirm that the ability of Vpu to block IκB degradation is linked to its ability to bind TrCP, demonstrating that Vpu indeed acts as a transdominant negative inhibitor of TrCP. Inhibition of IκB degradation by Vpu was found to severely impair TNF-α-induced NF-κB activation. Similarly, Vpu was found to significantly reduce HIV-induced activation of the NF-κB pathway. Taken together, these results suggest that Vpu has a transdominant negative effect on TrCP function that impairs both HIV- and cytokine-mediated activation of NF-κB.

EXPERIMENTAL PROCEDURES

Cells and Plasmids—Plasmids expressing the NL4-3 full-length molecular clone of HIV-1 or a Vpu-defective variant NL4-3/Udel have been described previously (1, 23). Construction of the Env-defective variant of pNL4-3, pNL43-K1, was previously described (24). The subgenomic viral expression plasmid pNL-A1 used here for the expression of Vpu was derived from a Vif-cDNA clone and encodes all other viral genes except *gag* and *pol* (25). The CD4 expression plasmid pHIV-CD4ΔBam and pCMV-CD4 expressing full-length CD4 under the transcriptional control of the HIV-1 long terminal repeat or the cytomegalovirus immediate early promoter, respectively, were described before (7). Plasmid pcDNA-TrCPΔF was derived from pcDNA-TrCP, encoding the wild type human βTrCP protein under the transcriptional control of the cytomegalovirus immediate early promoter (8). pcDNA-TrCPΔF bears a 148-amino acid deletion (residues 32–179) that includes the F-box domain described previously (8). CD4/Vpu chimeric proteins bearing the Vpu transmembrane and cytoplasmic domains fused to the CD4 ectodomain (CD4U and $CD4U_{2/6}$) were engineered with a structure similar to those previously described (26, 27). The chimeras were constructed in a two-step PCR as follows. First, two PCR fragments, encoding the CD4 ectodomain (amino acids 1–369) and Vpu (amino acids 2–81), respectively, were generated using pHIV-CD4ΔBam or pNL-A1 plasmid DNAs as PCR templates. The primary PCR products were designed such that the two fragments shared a 29-bp overlap, which allowed the two PCR products to anneal during the second PCR. The second PCR was performed using the outside primers from the first reaction and the two primary PCR products as templates. The resulting 1111-bp PCR product encoded the CD4 ectodomain fused to the Vpu TM and cytoplasmic domains. The final PCR product was digested with *Mfe*I and *Kpn*I (New England Biolabs, Beverly MA) and cloned into the corresponding sites in pHIV-CD4ΔBam. The pHIV-$CD4U_{2/6}$ carrying serine to alanine mutations at positions 52 and 56 of Vpu was generated in an analogous reaction except that pNL-A1/$U_{2/6}$ (6) plasmid DNA was used as template for the primary PCR. Expression of CD4U and $CD4U_{2/6}$ from these plasmids was Tat-dependent. For Tat-independent expression, CD4U and $CD4U_{2/6}$ were placed under the transcriptional control of the cytomegalovirus immediate early promoter by subcloning a 1559-bp *Eco*RI–*Kpn*I fragment from pHIV-CD4U or pHIV-$CD4U_{2/6}$ into pcDNA3.1(−) (Invitrogen, Carlsbad, CA).

For the construction of stable, inducible cell lines, CD4U and $CD4U_{2/6}$ chimeras were placed under the transcriptional control of a tetracycline-repressible promoter as follows: First, the multiple cloning site of the pTRE plasmid (CLONTECH, Palo Alto, CA) was modified by oligonucleotide-directed PCR mutagenesis. The multiple cloning site of the resulting vector, pTRS, contains 5′ *Sac*II, *Eco*RI, *Nhe*I, *Bsr*GI (compatible with *Acc*65I), *Afl*II, *Bam*HI, *Not*I, *Xho*I, and *Xba*I sites. Plasmids pTRS-CD4U and pTRS-$CD4U_{2/6}$ were constructed by inserting a 1542-bp *Eco*RI–*Acc*65I fragment from pHIV-CD4U or pHIV-$CD4U_{2/6}$, respectively, into the *Eco*RI–*Bsr*GI sites of pTRS.

For measuring NF-κB activity, the NF-κB indicator plasmid, pNFκB-Luc, was employed (Stratagene, La Jolla, CA). pNFκB-Luc expresses the luciferase gene under the control of an NF-κB-dependent minimal promoter containing five NF-κB binding sites.

Antibodies—For the detection of HIV-1-specific proteins, an HIV-positive patient serum reacting with all major HIV-1 proteins was used. A rabbit polyclonal antiserum directed against the cytoplasmic domain of Vpu (U2-3) was used for the identification of Vpu (28). The T4-4 rabbit polyclonal antibody, directed against the ectodomain of CD4, was obtained from the AIDS Research and Reference Reagent Program and was originally contributed by R. Sweet (29). The T4-Cy rabbit polyclonal antibody is directed against the cytoplasmic tail of the CD4 molecule and was previously described (30). Rabbit polyclonal antibodies to IκB (anti-IκB) and phosphorylated IκB (anti-P-IκB) were obtained from New England Biolabs.

Construction of Tetracycline-inducible CD4U and $CD4U_{2/6}$ Cell Lines—Approximately 2×10^6 HeLa Tet-off cells (CLONTECH, Palo Alto, CA) were transfected with 30 μg of pTRS-CD4U or pTRS-$CD4U_{2/6}$ plasmid DNAs along with 2 μg each of the hygromycin expression vector pTK-Hyg (CLONTECH) using a standard calcium-phosphate transfection protocol as described previously (30). Cells were maintained in G418 (1 mg/ml), tetracycline (100 ng/ml), and hygromycin (200 μg/ml) selection medium for 2 weeks, at which time individual drug-resistant clones were harvested. Individual clones were tested for their ability to express CD4U or $CD4U_{2/6}$ in an inducible fashion by cultivating cells in Dulbecco's modified Eagle's medium containing 10% tetracycline-free fetal calf serum (CLONTECH) for 48 h. CD4U and $CD4U_{2/6}$ expression was assessed by Western blotting as described below using the U2-3 anti-Vpu antibody. Following initial selection, cells were maintained in the presence of the tetracycline analogue doxycycline (10 ng/ml).

Immunoblotting—Cells (5×10^6) were lysed in 200 μl of CHAPS lysis buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 100 mM NaCl, 0.5% CHAPS) and 25 μl of DOC buffer (2% deoxycholate in CHAPS lysis buffer) supplemented with a protease/inhibitor mixture (Complete™; Roche Molecular Biochemicals). Insoluble material was removed by centrifugation, and the supernatant was combined with an equal volume of sample buffer and boiled. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon membranes. Following transfer, membranes were blocked for 30 min with 5% dry milk in TN-T buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.3% (v/v) Tween 20). Membranes were briefly rinsed with TN-TN wash buffer (0.05% IGEPAL CA-630 in TN-T buffer) and then incubated for 1 h with the primary antibody in 3% bovine serum albumin in TN-T. Blots were then washed once each for 5 min with TN-TN and TN-T and then reacted for 45 min in 3% bovine serum albumin/TN-T with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech). Membranes were washed twice each with TN-TN and TN-T (5 min each wash). Proteins were visualized by ECL (Amersham Pharmacia Biotech). Bands were quantified after densitometric scanning of the films using Fuji MacBAS software.

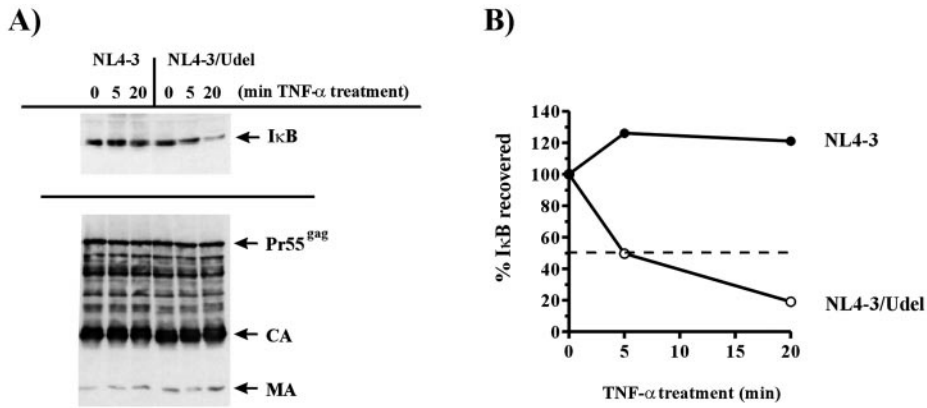

FIG. 1. **Vpu inhibits TNF-α-induced degradation of IκB in infected A3.01 cells.** A3.01 cells were electroporated with pNL4-3 (NL4-3) or the Vpu-deficient pNL4-3/Udel (NL4-3/Udel) plasmid DNA. *A*, seven days postinfection, cells were harvested, and equal fractions were treated with TNF-α (20 ng/ml) for 5 or 20 min. A control sample (0 min) was left untreated. Cells were lysed in detergent buffer containing protease inhibitors. Equal aliquots of each cell lysate were separated by 12.5% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon membranes. IκB-α was revealed by immunoblotting using a polyclonal antibody to IκB-α followed by ECL (*top panel*). The same membrane was subsequently reacted with an HIV-positive patient serum to detect viral proteins in the lysates (*bottom panel*). Proteins are identified on the *right*. *B*, bands corresponding to IκB-α were quantified using the Fuji MacBAS software and plotted as a percentage of the corresponding uninduced sample.

Analysis of NF-κB Activity: Luciferase Assay—One day prior to transfection, target cells were placed into six-well plates (approximately 2×10^5 cells/well). Cells were transfected using FuGENE (Roche Diagnostics Corp.) or LipofectAMINE (Life Technologies, Inc.) transfection reagents. In general, a total of 4–5 μg of plasmid DNA (1 μg of pNFκB-luc reporter plasmid plus 3–4 μg of test plasmid(s) or filler DNA (pcDNA3.1(−)) was used for transfection. Cells were harvested 20–24 h after transfection. Where appropriate, cells were treated with TNF-α (20 ng/ml; Calbiochem) for 5–20 min prior to cell lysis. For measuring NF−κB activation by luciferase assay, TNF-α-containing medium was replaced with normal tissue culture medium after an initial 15-min stimulation followed by a 5-h incubation. Cell extracts were prepared by lysis in 1× Reporter Lysis Buffer (Promega, Madison, WI). Cell lysates were normalized for protein content, and luciferase activity was determined using the Promega Luciferase Assay System. Samples were analyzed using an Optocomp II Luminometer (MGM Instruments, Hamden, CT).

Electroporation of A3.01 Cells—Productive HIV-1 infection was initiated in CD4-positive A3.01 cells by electroporation of pNL4-3 or pNL4-3/Udel plasmid DNA. Approximately 5×10^6 cells in exponential growth phase were electroporated with 5 μg of DNA in duplicate using a Bio-Rad Gene Pulser II with a capacitance extender set at 0.3 kV and 975 microfarads. Electroporated cells were immediately transferred to a 12-well culture plate containing 1×10^6 fresh A3.01 cells in complete RPMI 1640 medium (total volume, 2 ml). Ninety percent of the culture medium was replaced 12 h postelectroporation, and aliquots were collected every 2 days thereafter for the purpose of monitoring the infection kinetics by reverse transcriptase assay.

RESULTS

Vpu Inhibits the TNF-α-induced Degradation of IκB in HIV-infected T Cells—In a first set of experiments, we assessed the impact of Vpu expression on the TNF-α-induced degradation of IκB in HIV-1-infected T cells. Infections were initiated in the CD4+ A3.01 cell line by electroporation of plasmid DNA encoding the molecular clone NL4-3 or its Vpu-defective variant NL4-3/Udel. Virus replication was monitored by measuring the viral reverse transcriptase activity in the infected cultures (data not shown). Cells were harvested 7 days after electroporation near the time of peak virus production, and equal aliquots were subjected to treatment with TNF-α for 5 or 20 min or were left untreated (0 min). Cell lysates were then analyzed by immunoblotting with an IκB-specific antibody (Fig. 1*A*, *top panel*). To control for differences in infection rates and relative protein concentrations, the same blot was subsequently reacted with an HIV-positive patient serum (Fig. 1*A*, *bottom panel*). IκB-specific bands were quantified and plotted as a percentage of the amount recovered before TNF-α stimulation (Fig. 1*B*, time 0). The response of infected A3.01 cells to treatment with TNF-α, as measured by the degradation of IκB-α, was markedly different in cells infected with wild type *versus* Vpu-deficient HIV-1 (Fig. 1*A*, *top panel*). In fact, in cells infected with the Vpu-defective virus, TNF-α stimulation resulted in degradation of over 80% of total IκB within 20 min of treatment (Fig. 1*B*, NL4-3/Udel). In striking contrast, TNF-α treatment of cells infected with wild type NL4-3 failed to induce IκB degradation during the 20-min induction time (Fig. 1*B*, NL4-3). This difference in the cellular response to TNF-α treatment following infection with wild type and Vpu-defective viruses could not be attributed to different infection efficiencies, since the levels of viral proteins expressed in the two cultures at the time of the experiment were virtually identical (Fig. 1*A*, *bottom panel*). These data therefore suggest that accumulation of Vpu in T cells during a normal productive HIV-1 infection blocks IκB degradation following TNF-α stimulation.

Vpu Inhibits Degradation of Phosphorylated IκB-α in a Dose-dependent Manner—We next examined whether the presence of Vpu alone could account for the inhibition of IκB-α degradation observed in Fig. 1. Presumably due to the presence of cryptic splice signals within the Vpu-coding region, we were unable to express detectable levels of Vpu from Tat- and Rev-independent vector systems (data not shown). Interestingly, we found that insertion of the fully spliced extracellular domain of human CD4 upstream of the Vpu coding sequence resulted in efficient and stable expression of the chimeric protein. We made use of this phenomenon by creating chimeric molecules between the CD4 ectodomain and wild type Vpu or a Vpu mutant (S52N/S56N) previously shown to be unable to interact with human βTrCP (8). The resulting constructs, termed CD4U and $CD4U_{2/6}$, respectively, showed high levels of Rev-independent protein expression in HeLa cells (data not shown). To verify that CD4U and $CD4U_{2/6}$ are functionally comparable with Vpu and $Vpu_{2/6}$, respectively, we first assessed the ability of the chimeras to induce degradation of CD4 (Fig. 2*A*). Due to the absence of the CD4 cytoplasmic domain, which contains sequences critical for TrCP-dependent degradation, CD4U chimeras were expected to be resistant to TrCP-dependent degradation. Degradation of CD4 was measured by comparing steady state levels of CD4 in the presence or absence of Vpu, $Vpu_{2/6}$, CD4U, or $CD4U_{2/6}$. HeLa cells were transfected with equal amounts of pHIV-CD4ΔBam (CD4) in combination with pHIV-CD4U (CD4U), pHIV-$CD4U_{2/6}$ ($CD4U_{2/6}$), pNL-A1 (Vpu), or pNL-A1/$U_{2/6}$ ($Vpu_{2/6}$), as indicated in Fig. 2*A*. Therefore, ex-

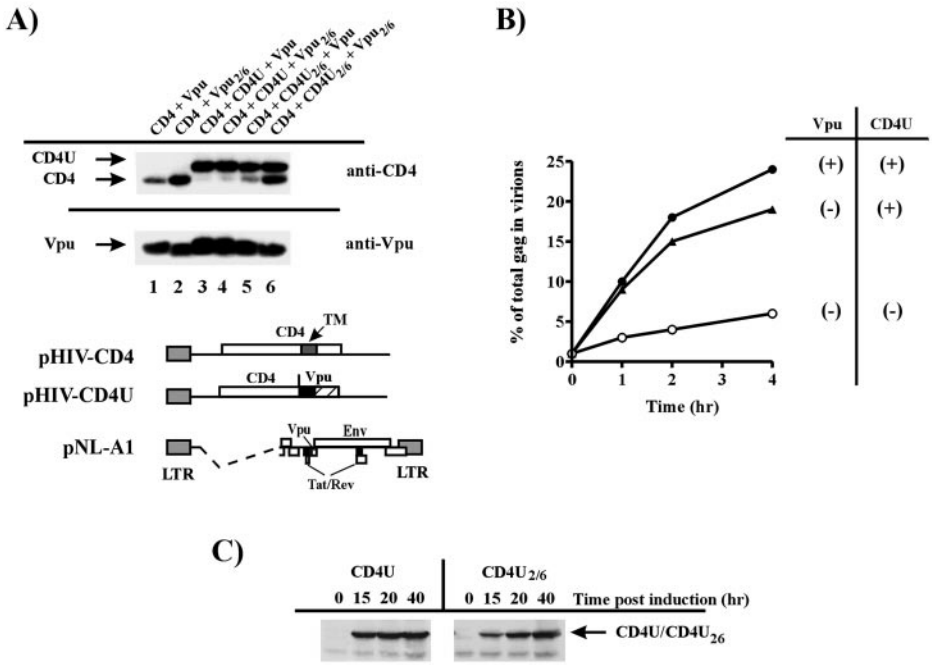

FIG. 2. **Molecular characterization of the CD4U and CD4U$_{2/6}$ chimeric proteins.** *A*, CD4U and CD4U$_{2/6}$ are functionally equivalent to Vpu with respect to their ability to induce CD4 degradation. Vpu and Vpu$_{2/6}$ expression in this experiment was dependent on Tat and Rev, both of which are encoded by the pNL-A1 construct. Expression of CD4 CD4U, and CD4U$_{2/6}$ was Tat-dependent as well. HeLa cells were transfected with 5 μg of pHIV-CD4ΔBam and equimolar amounts of plasmids expressing Vpu (pNL-A1), Vpu$_{2/6}$ (pNL-A1/U$_{2/6}$), CD4U (pHIV-CD4U), or CD4U$_{2/6}$ (pHIV-CD4U$_{2/6}$). Cells were harvested 24 h post transfection. Detergent extracts were loaded on a 12.5% gel and transferred to Immobilon membranes. Immunoblotting was performed as in Fig. 1 using the ECL system. CD4 and CD4U proteins present on the membrane were revealed using a mixture of the T4-4 and T4-Cy polyclonal antisera (anti-CD4). Wild type Vpu and the TrCP-binding mutant, Vpu$_{2/6}$, were detected on the same blot using the Vpu-specific U2-3 antibody (anti-Vpu). The positions of CD4, Vpu, and the slower migrating CD4U chimera are indicated on the *left*. The plasmid constructs used in this experiment are schematically shown at the *bottom*. *B*, CD4U supports efficient virus release. HeLa cells were transfected with Vpu-defective (*Vpu* (−)) or Vpu-expressing (*Vpu* (+)) variants of pNL4-3/K1 in the presence or absence of pHIV-CD4U (*CD4U*) as indicated on the *right*. The effect of CD4U on virus release was determined by pulse/chase analysis. Cells were pulse-labeled for 30 min in the presence of [^{35}S]methionine and chased for up to 4 h. At each time point, viral proteins present in the cell and medium fractions were recovered by centrifugation and lysed in detergent buffer. Viral proteins were immunoprecipitated with an HIV-positive human serum, separated by SDS-polyacrylamide gel electrophoresis, and visualized by fluorography (not shown). HIV Gag proteins were quantified, and particle release was calculated as the ratio of viral proteins detected in the medium over the total amount of viral proteins present in both the cell and medium fractions. *C*, inducible expression of CD4U and CD4U$_{2/6}$. Stable HeLa cell lines expressing tetracycline-inducible CD4U or CD4U$_{2/6}$ chimeras were constructed as described under "Experimental Procedures." For the experiment shown here, cells (3×10^6 each) were seeded into 25-cm^2 tissue culture flasks (1 flask/time point). Cells were grown in the absence of doxycycline for 15, 20, or 40 h before cells were harvested. An uninduced control culture (0 h) was grown in the presence of doxycycline for 15 h before cells were harvested. Detergent lysates from each time point were analyzed by immunoblotting for CD4U or CD4U$_{2/6}$ expression using a CD4-specific antibody. The position of CD4U and CD4U$_{2/6}$ in the gel is marked on the *right*.

pression of CD4 and the chimeric CD4/Vpu molecules was dependent on the expression of Tat from pNL-A1 or pNL-A1/U$_{2/6}$. Cell lysates were prepared 24 h after transfection and analyzed by immunoblotting with antibodies to CD4 (Fig. 2*A*, anti-CD4) or Vpu (Fig. 2*A*, anti-Vpu). Expression of wild type Vpu from pNL-A1 (*lane 1*) significantly reduced the steady state levels of CD4 when compared with cells expressing the TrCP binding mutant Vpu$_{2/6}$ (*lane 2*) although comparable levels of Vpu protein were expressed (*lanes 1* and *2*, anti-Vpu). Importantly, coexpression of CD4U (*lanes 3* and *4*) resulted in almost complete depletion of the CD4 steady state levels even in Vpu$_{2/6}$-expressing cells (*lane 4*). This suggests that CD4U is able to induce degradation of CD4 with similar efficiency than authentic wild type Vpu. Coexpression of CD4U$_{2/6}$ did not affect the ability of wild type Vpu to induce CD4 degradation (compare *lanes 1* and *5*, CD4). As expected, coexpression of Vpu$_{2/6}$ and CD4U$_{2/6}$ (*lane 6*) did not affect CD4 levels and resulted in CD4 steady state levels similar to those observed in cells expressing Vpu$_{2/6}$ only (compare *lanes 2* and *6*). These results demonstrate that CD4U but not CD4U$_{2/6}$ has the ability to induce CD4 degradation. These data also indicate that CD4U has retained the ability to interact with βTrCP, a necessary event in the process of CD4 degradation.

We also tested the ability of CD4U to regulate virus release. To avoid an interference of HIV-1 Env with CD4U function due to the formation of intracellular complexes between the Env and CD4 ectodomains (31), the Env-defective variant of pNL4-3, pNL43-K1, was used in this experiment (Fig. 2*B*). HeLa cells were transfected with Vpu-defective (Vpu (−)) or Vpu-expressing (Vpu (+)) variants of pNL4-3/K1 in the presence of absence of pHIV-CD4U (CD4U), and the effect of CD4U on virus release was determined by pulse/chase analysis as described in the legend to Fig. 2*B*. In the absence of Vpu (Fig. 2*B*, Vpu (−)) expression of CD4U in *trans* increased virus release by more than 3-fold (Fig. 2*B*, Vpu (−), CD4U (+)). Coexpression of Vpu and CD4U showed only little synergistic effect on particle release, indicating that CD4U alone is sufficient for near maximal virus release (Fig. 2*B*, Vpu (+), CD4U (+)). These data demonstrate that CD4U can indeed enhance the release of virus particles in a manner similar to wild type Vpu. Taken together, our results show that CD4U and CD4U$_{2/6}$ are functionally equivalent to Vpu and Vpu$_{2/6}$, respectively, both with respect to CD4 degradation and enhancement of virus release.

To directly address the effect of Vpu on IκB metabolism, we constructed stable cell lines expressing CD4U and CD4U$_{2/6}$

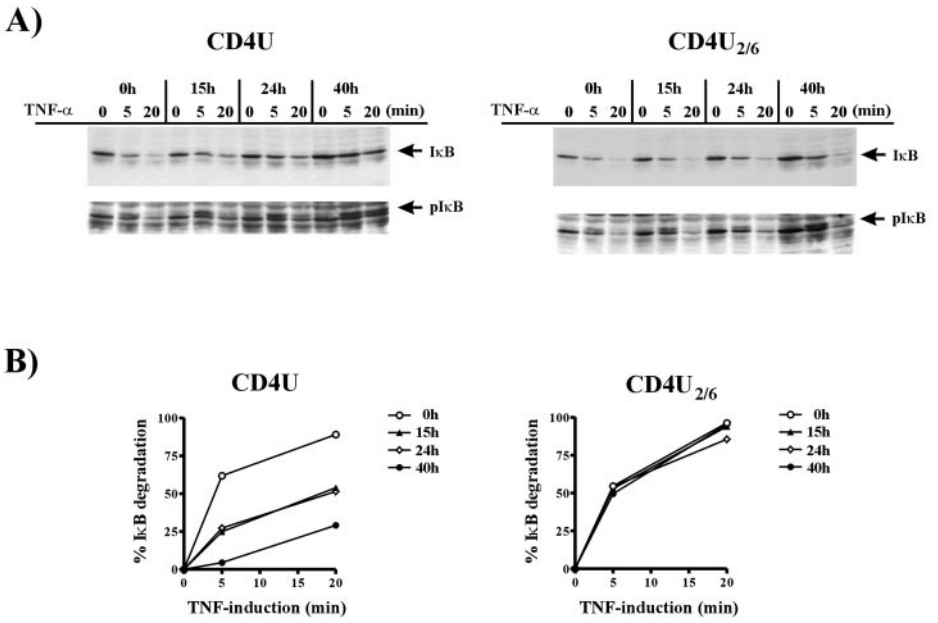

FIG. 3. **Vpu inhibits the SCF^{TrCP}-mediated degradation of phosphorylated IκB-α in a dose-dependent manner.** *A*, CD4U and CD4U$_{2/6}$ cell lines were induced by the removal of doxycycline as described for Fig. 2*C*. Cells were harvested at the indicated time points, washed once in phosphate-buffered saline, and divided into three equal fractions. One fraction was left untreated (0 min). Cells from the other two fractions were treated with TNF-α (20 ng/ml) for 5 or 20 min, respectively. Cell lysates were then subjected to immunoblot analysis using antibodies to total IκB (IκB) or phospho-IκB (*pIκB*) followed by ECL. *B*, IκB-α-specific bands were quantified as in Fig. 1*B* and used for the calculation of the degradation kinetics (percentage degraded relative to time 0).

under the control of an inducible tetracycline-repressed promoter (Tet-off). Individual cell clones were screened for their ability to express the chimeric proteins in an inducible fashion by removal of the doxycycline inhibitor and assessment of CD4U and CD4U$_{2/6}$ expression over time by immunoblotting using a CD4-specific antiserum. As shown in Fig. 2*C*, the two clones selected express CD4U and CD4U$_{2/6}$ with similar efficiency.

The inducible CD4U and CD4U$_{2/6}$ lines were used to assess the impact of Vpu on endogenous IκB turnover in the absence of other viral proteins. This was done by measuring the TNF-α-mediated degradation of IκB at various times following induction of CD4U and CD4U$_{2/6}$. Cells were grown in 25-cm^2 tissue culture flasks in the presence (0 h) or absence (15–40 h) of doxycycline. Cells were harvested at the indicated time points, and equal aliquots were stimulated with TNF-α (20 ng/ml) for 0, 5, or 20 min (Fig. 3*A*). Cell lysates were then analyzed by immunoblotting first with antibodies to total IκB (Fig. 3*A*, IκB), followed by reaction with antibodies specific for phosphorylated IκB (Fig. 3*A*, *pIκB*). IκB-specific bands were quantified by densitometric scanning, and the relative amounts of IκB remaining at the indicated times after TNF-α stimulation were used to calculate the percentage of IκB degradation as a function of time (Fig. 3*B*). As expected, TNF-α treatment of doxycycline-repressed CD4U or CD4U$_{2/6}$ cells resulted in the rapid degradation of IκB (Fig. 3*A*, IκB, 0 h). This was accompanied in both cases by the transient appearance 5 min after TNF-α treatment of a band corresponding to phosphorylated IκB (Fig. 3*A*, *pIκB*, 0 h). Quantitation of total IκB in uninduced cells revealed that in both cell lines 90–95% of the total IκB protein was degraded within 20 min of TNF-α treatment (Fig. 3*B*, 0 h). Thus, both cell lines show a similar response to TNF-α stimulation and have the same intrinsic ability to phosphorylate and degrade IκB. Induction of CD4U$_{2/6}$ expression following removal of doxycycline did not interfere with TNF-α response, and the kinetics of IκB degradation following TNF-α treatment remained virtually unchanged even after 40 h of CD4U$_{2/6}$ induction (Fig. 3, *A* and *B*, CD4U$_{2/6}$). In striking contrast, induction of CD4U resulted in an increasing inhibition of TNF-α-induced degradation of IκB. Forty hours after CD4U induction, only about 25% of total IκB was degraded within the 20 min of TNF-α treatment (Fig. 3*B*, CD4U). Thus, intracellular accumulation of CD4U resulted in the dose-dependent inhibition of TNF-α-induced degradation of IκB. Interestingly, the presence of CD4U had no apparent effect on the TNF-α-induced phosphorylation of IκB (Fig. 3*A*, *pIκB*). In fact, phospho-IκB appeared to accumulate in response to rising levels of CD4U in the cells. This suggests that Vpu does not affect TNF-α-induced activation of the IκB kinase but instead blocks the subsequent TrCP-dependent degradation of phosphorylated IκB-α.

CD4U-mediated Inhibition of IκB Degradation Leads to Reduced NF-κB Transcriptional Activity—The results shown in Figs. 1 and 3 demonstrate a pronounced inhibition of IκB degradation following cytokine stimulation in Vpu or CD4U-expressing cells. To assess the impact of this phenomenon on the transcriptional activity of NF-κB, we examined the effect of CD4U or CD4U$_{2/6}$ on NF-κB transcriptional activity using a luciferase reporter gene. The HeLa CD4U- and CD4U$_{2/6}$-inducible cell lines were transfected in triplicate with the pNFκB-Luc reporter plasmid, and CD4U or CD4U$_{2/6}$ protein synthesis was induced by removal of doxycycline. At 48 h postinduction, cells were incubated in the presence or absence of TNF-α (10 ng/ml) for 15 min. The cytokine was then removed, and cells were cultured for an additional 6 h to allow for luciferase synthesis. The control cultures were treated in the same fashion but remained in the presence of doxycycline throughout the experiment. As shown in Fig. 4*A*, TNF-α treatment resulted in a comparable activation of NF-κB in uninduced (Dox (+)) CD4U and CD4U$_{2/6}$ cell lines. Induction of CD4U$_{2/6}$ expression (CD4U$_{2/6}$, Dox (−)) did not affect the TNF-α response in these cells and resulted in NF-κB activation similar to that observed in uninduced cells (compare CD4U$_{2/6}$, Dox (+), and Dox (−)). In contrast, induction of CD4U expression (CD4U, Dox (−)) led to a 3.5-fold reduction in the amount of luciferase activity measured (compare CD4U, Dox (+), and Dox (−)). Interestingly, CD4U but not CD4U$_{2/6}$ also decreased by 3.5-fold the basal level of NF-κB activity observed in the absence of TNF-α stimulation (Fig. 4*A*, *TNF* (−)). Taken together, these results show that inhibition of IκB degradation by Vpu in infected T cells or stably transfected HeLa cells (Figs. 1 and 3) has a direct negative impact on transcriptionally active NF-κB.

To further support the notion that the effect of Vpu on NF-κB activity is caused by the inhibition of TrCP-dependent IκB degradation, we compared the effects of CD4U and a trans-dominant negative mutant of TrCP, TrCPΔF (8), on TNF-α-

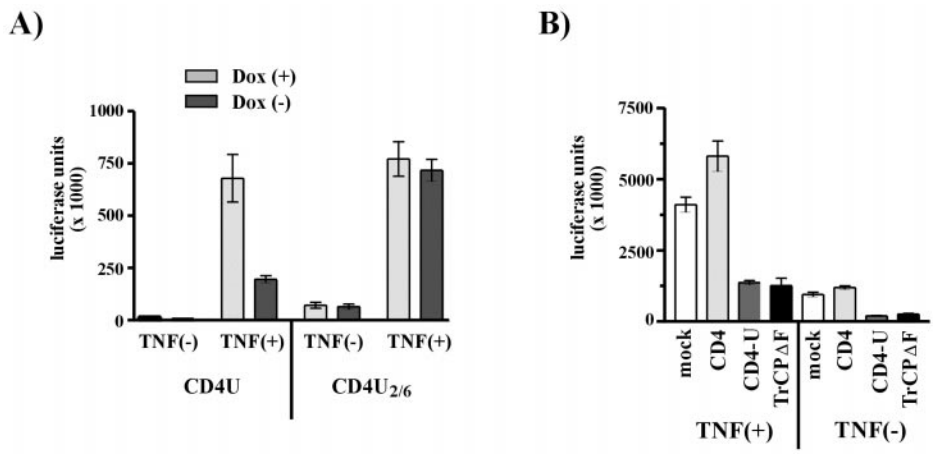

FIG. 4. **Vpu interferes with TrCP to inhibit TNF-α-induced activation of NF-κB.** *A*, inducible HeLa-CD4U and $CD4U_{2/6}$ cell lines were transfected with 1 μg of pNFκB-Luc and maintained in the presence or absence of doxycycline (*Dox* (+) or *Dox* (−)) for 48 h. Cells were then incubated in the presence or absence of TNF-α (10 ng/ml) for 15 min. Cells were cultured in the absence of TNF-α for an additional 6 h, and the relative amount of luciferase activity was obtained from triplicate measurements. *B*, HeLa cells were transfected in triplicates with 1 μg of reporter plasmid (pNFκB-Luc) and 4 μg of either pcDNA3.1(−) (*mock*), pCMV-CD4 (*CD4*), pcDNA-CD4U (*CD4U*), or pcDNA-TrCPΔF (*TrCPΔF*). Cells were harvested 24 h post-transfection. Equal fractions of cells were either treated with 10 ng/ml of TNF-α (TNF(+)) for 15 min or left untreated (TNF(−)) and cultured for an additional 5 h at 37 °C. Cell lysates were normalized for equal protein content, and luciferase activity was determined. *Error bars* reflect the S.D.

induced NF-κB activity (Fig. 4*B*). HeLa cells were each transfected with pNFκB-Luc reporter plasmid along with empty pcDNA3.1(−) vector DNA (mock), pCMV-CD4 (CD4), pcDNA-CD4U (CD4U), or pcDNA-TrCPΔF (TrCPΔF) expressing a TrCP F-box deletion mutant, which was previously found to have a transdominant negative effect on both CD4 and IκB degradation (8, 13). At 24 h post-transfection, one set of cells was treated with TNF-α for 15 min followed by a 5-h incubation at 37 °C (Fig. 4*B*, *TNF* (+)). A second set of cells was left untreated (*TNF* (−)). Cells were lysed and assayed for luciferase activity. As predicted, TNF-α treatment of mock-transfected cells resulted in a significant induction of NF-κB-driven luciferase expression during the 5-h incubation period relative to the unstimulated culture (Fig. 4*B*, *mock*). Similarly, expression of wild-type CD4 did not affect the cellular TNF-α response and resulted in an increase in luciferase activity comparable with the mock control (CD4). In contrast, expression of CD4U significantly reduced luciferase expression, further confirming that the Vpu component of the CD4U chimera was responsible for the repression of NF-κB activation described above. Consistent with the results from Fig. 4*A*, CD4U expression in unstimulated cells reduced the basal activity of NF-κB. The effect of CD4U was strikingly similar to that of the transdominant negative TrCP mutant, suggesting that the two proteins act at a common step in the degradation cascade, *i.e.* the binding of TrCP to phosphorylated IκB (Fig. 4*B*, TrCPΔF). The similarity between the inhibitory effects exerted by CD4U and TrCPΔF further supports our conclusion that CD4U acts as a competitive inhibitor of the SCF^{TrCP}.

Vpu Modulates HIV-1-induced NF-κB Activation—Previous reports indicate that HIV-1 infection of cells can result in a constitutive activation of NF-κB (32–34). Our data indicate that Vpu inhibits NF-κB activation in response to TNF-α treatment. We therefore addressed whether this effect of Vpu is also observed in the context of HIV-mediated activation of NF-κB. In a first set of experiments, HeLa cells were transfected with the pNFκB-luc indicator plasmid together with subgenomic constructs expressing all HIV-1 proteins except Gag and Pol (pNL-A1). As seen in Fig. 5*A*, transfection of pNL-A1 led to a moderate 3-fold activation of NF-κB, as compared with the mock control expressing no viral protein. However, expression of pNL-A1/Udel, a Vpu-deficient variant of pNL-A1, led to a much higher 14-fold enhancement of NF-κB activity. In addition, in the presence of the βTrCP-binding mutant of Vpu, $Vpu_{2/6}$ (pNL-A1/$U_{2/6}$), NF-κB activity was similar to that observed following transfection of pNL-A1/Udel. Very similar results were obtained using full-length HIV-1 constructs (Fig. 5*B*). These data indicate that HIV-1 encodes proteins with the intrinsic ability to enhance NF-κB activity but that Vpu acts as a negative modulator of that activity. Vpu is therefore capable of interfering not only with TNF-α-mediated but also with HIV-1-induced activation of NF-κB. Moreover, Vpu acts on both stimuli through the same mechanism, since in both cases mutation of the TrCP binding site abolished the inhibitory activity of Vpu.

DISCUSSION

HIV-1 is a human retrovirus that induces long term chronic infection in affected individuals. The virus is highly pathogenic and leads to the complete destruction of the immune system in the vast majority of patients. Disease progression is controlled by a complex interplay of viral and host factors, in particular cytokines that regulate the immune response (for a review, see Ref. 35). Chronic HIV infection results in the deregulation of numerous cellular functions, including cytokine production, which can either be increased or decreased (reviewed in Ref. 36). In HIV-1-positive individuals, expression of the proinflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8 was found to be increased, especially at late stages of the disease (37–42). Conversely, expression of the immunoregulatory cytokines IL-2 and IL-12 was gradually lost over time (43, 44).

As one of the key regulators of cytokine expression, NF-κB is likely to play a role in the perturbation of cytokine production observed in HIV-infected individuals. In fact, three main mechanisms have been proposed to account for the observed persistent activation of NF-κB in HIV-1-infected cells: hyperphosphorylation and degradation of IκB-α caused by the constitutive activation of the IκB kinase (32, 33), inhibition by nuclear IκB-β of the IκB-α-mediated dissociation of NF-κB/DNA complexes, and, more recently, modulation of the NF-κB-interacting p300 transcriptional co-activator (45). Several HIV proteins have been shown to directly or indirectly affect NF-κB regulation. These include Tat, envelope gp120, and the accessory protein Vpr (42, 45–49). We now report that the HIV-1-specific

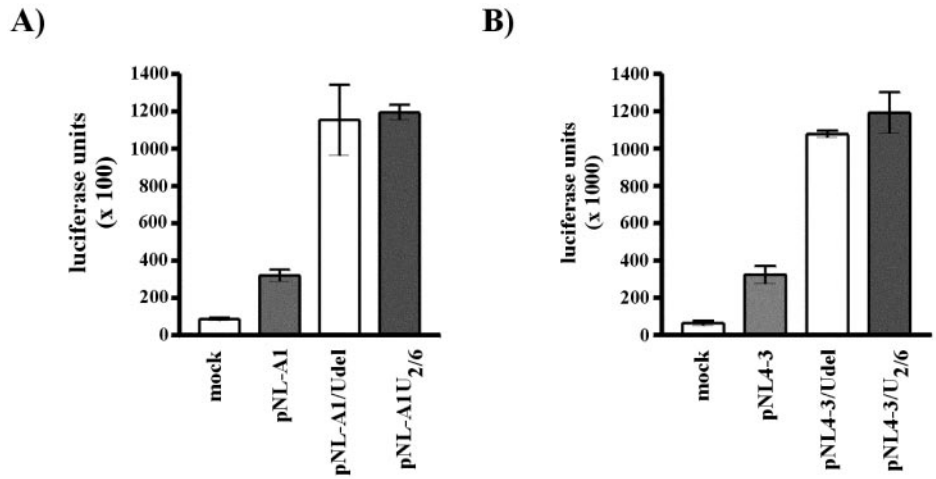

FIG. 5. **Vpu modulates HIV-1-induced NF-κB activation.** *A*, HeLa cells were transfected in quadruplicate sets with each 1 μg of reporter plasmid (pNFκB-Luc) and 4 μg of either pcDNA3.1(−) (*mock*) or pNL-A1-based subgenomic constructs as indicated. Cells were harvested 24 h post-transfection. Luciferase activity was determined on lysates normalized for equal protein content. *Error bars* reflect the S.D. *B*, HeLa cells were transfected in triplicate sets with 1 μg of reporter plasmid (pNFκB-Luc) and 4 μg of pcDNA3.1(−) (*mock*) or the NL4-3 full-length molecular clone constructs as indicated. Cells were harvested 24 h post-transfection, and luciferase activity was determined as described above. *Error bars* reflect the S.D.

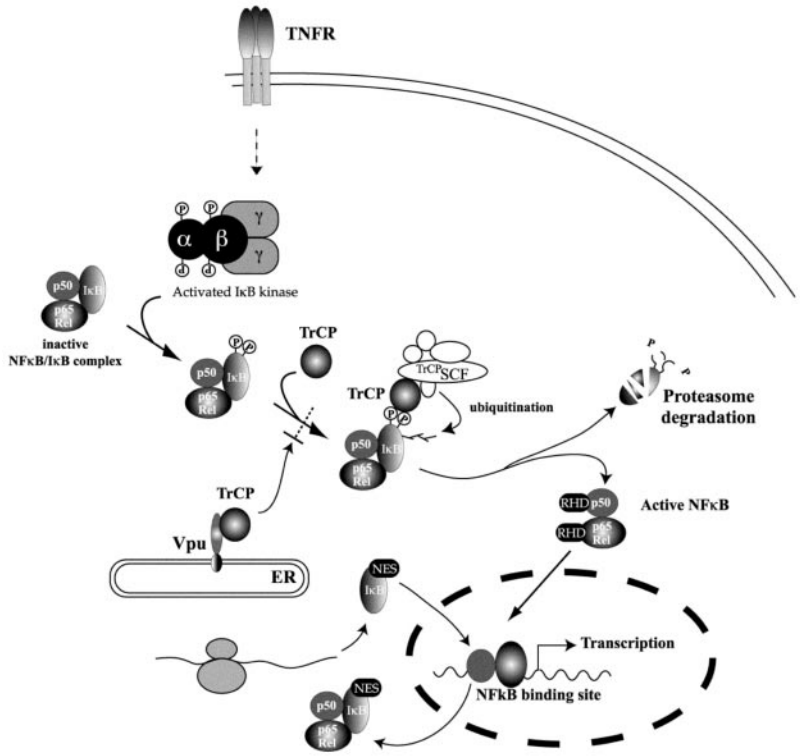

FIG. 6. **Model of Vpu interference with cellular TrCP function.** The ability of Vpu to competitively inhibit TrCP function is based on the fact that Vpu is constitutively phosphorylated at two serine residues (Ser^{52} and Ser^{56}), which are part of a TrCP binding motif. However, unlike normal cellular substrates of TrCP, Vpu is not targeted for degradation following binding to TrCP. Therefore, the intracellular accumulation of Vpu during the course of infection leads to a progressive inhibition of TrCP-dependent degradation of IκB and thus to a gradual inhibition of NF-κB. Details of the model are explained under "Discussion."

Vpu protein is also involved in the regulation of NF-κB activity by acting as a negative modulator of TNF-α- and HIV-mediated hyperactivation of NF-κB. Our data suggest that Vpu functions both in HIV-1-infected T cells and in transfected HeLa cells by acting as a competitive inhibitor of βTrCP, thereby preventing the efficient degradation of phosphorylated IκB. Although NF-κB activation was chosen to illustrate the effect of Vpu on TrCP function, it is likely that this activity of Vpu has an even broader impact on cell function. Indeed, one would expect that Vpu can perturb with similar efficiency the degradation of other substrates of TrCP such as β-catenin (50).

The majority of the currently known HIV-1 isolates encode a complete *vpu* gene (51). However, a number of HIV-1 isolates such as HxB2 and HTLVIIIB/LAI/BRU, which have been widely used in tissue culture studies, do not encode a functional Vpu protein (51). It is therefore conceivable that the regulatory role of Vpu could have been overlooked in cases where recombinant protein, subgenomic expression vectors, or Vpu-deficient viruses were used (32–34, 48, 52–54). Thus, our finding in the present study that Vpu negatively regulates the activation of NF-κB does not necessarily conflict with the previously reported activation of NF-κB in HIV-infected cells. Indeed, it is clear from our own results in both HeLa and A3.01 cells that the expression of full-length HIV-1 does lead to a net increase in NF-κB activity despite the presence of Vpu. However, this increase in NF-κB activity is small compared with the activation seen in the absence of Vpu (Fig. 5).

The effect of Vpu on NF-κB is dose-dependent and is mediated through inhibition of the TrCP-dependent degradation of IκB-α. Unlike most other viral proteins, Vpu is not packaged into virions or secreted but gradually accumulates in the cell. As a result, Vpu-mediated inhibition of NF-κB activation can be expected to be minor early in infection but to become more and more pronounced as the viral life cycle progresses. Nevertheless, our data obtained in HIV-infected T-cells (Fig. 1) or CD4U-inducible cell lines (Fig. 3) suggest that even when maximal amounts of Vpu are expressed, total inhibition of IκB degradation is not achieved, and NF-κB activity is not com-

pletely inhibited (Fig. 4). There are several possible explanations for this observation. First, it is possible that degradation of IκB is regulated by redundant cellular mechanisms. For example, TrCP2, a WD-F-box protein closely related to βTrCP, was recently found to target IκB for degradation (55). Although unlikely, we cannot formally rule out the possibility that the residual NF-κB activity observed in the presence of Vpu is due to an inability of the latter to bind and interfere with the function of TrCP2. Second, it is possible that due to its location in cellular membranes, Vpu is unable to prevent the nuclear degradation of newly synthesized IκB (56) and therefore might be unable to block the sustained activation of a small pool of NF-κB. Finally, it is possible that NF-κB can be activated through TrCP-independent mechanisms, such as the reported dissociation of IκB-NF-κB complexes following tyrosine phosphorylation of IκB (57).

Despite the fact that Vpu is unlikely to completely inhibit NF-κB activity *in vivo*, its effects on cell function could nevertheless be profound. Indeed, studies performed in transgenic mice expressing a dominant negative variant of IκB-α indicate that T- and B-cell development can be dramatically perturbed when levels of active NF-κB are lowered below a critical physiological threshold (58, 59). Also, there is a body of evidence indicating that NF-κB plays a central role in regulating cellular apoptosis (reviewed in Ref. 22). However, it is still debated whether HIV-1 infection and the ensuing constitutive activation of NF-κB can effectively protect cells from apoptosis. In some cases, activation of NF-κB has been shown to prevent the natural tendency of HIV to induce apoptosis (60), while it is also well documented that HIV infection can prime cells for apoptosis (61, 62). The complex interplay between NF-κB, TNF-α, PKR, and other factors determines whether or not a cell will be able to overcome an apoptotic signal (22). Nevertheless, it is possible that the gradual inhibition of NF-κB activity by Vpu during the course of an infection contributes to HIV-induced apoptosis. This notion is supported by the recent findings that NF-κB confers resistance against Fas-mediated apoptosis (63) and that Vpu increases susceptibility of human immunodeficiency virus type 1-infected cells to Fas killing (64). Based on the results described in the current study, we propose the model outlined in Fig. 6. In cells expressing Vpu, phosphorylation of IκB by various stimuli does not lead to the normal degradation of IκB and activation of NF-κB transcriptional activity. This is due to the fact that IκB degradation requires the activity of βTrCP, which, in Vpu-expressing cells, is competitively inhibited through interactions with the cytoplasmic tail of Vpu. Normal cellular substrates of βTrCP are rapidly ubiquitinated and degraded, thereby releasing βTrCP from the complex. In contrast, Vpu is not targeted for degradation but instead accumulates in virus-producing cells and forms stable complexes with βTrCP. Both the accumulation of Vpu in virus-producing cells and the stability of Vpu-TrCP complexes are likely to contribute to the efficiency of Vpu-mediated inhibition of βTrCP activity, which becomes more and more pronounced in late stages of virus replication.

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